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TGF- β and stem cell factor regulate cell proliferation in the proximal stem cell niche

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Abstract

BACKGROUND—Stem cells are located in specific regulatory environments termed niches, which modulate the survival and proliferation of the cells through a variety of both mitogenic and inhibitory cytokines. In the murine prostate, stem cells are located in the proximal region of prostatic ducts. We examined the regulation of murine prostate cells in the stem cell niche by transforming growth factor beta (TGF- β) and stem cell factor (SCF).

METHODS—Prostate cells from the proximal and distal regions of prostatic ducts were cultured in the presence and absence of TGF- β and SCF, both on collagen-coated wells and in collagen gels. Cell growth on collagen was assessed by determining cell number. Cell growth in collagen gels was quantified by determining the number, size and complexity of prostatic ducts. The basal and luminal phenotype of the cells was determined by immunohistochemistry.

RESULTS—Endogenous TGF- β inhibited proliferation and promoted differentiation of proximal cells towards a luminal phenotype. It also inhibited duct-forming capacity and promoted differentiation of prostatic ducts towards a luminal phenotype. Addition of SCF enhanced proximal cell proliferation on collagen-coated wells and duct formation in collagen gels. Proliferation was further increased by ablation of endogenous TGF- β .

CONCLUSION—Proliferation and the basal/luminal cell composition of cells isolated from the proximal region of prostatic ducts, the stem cell niche, is regulated in part by opposing effects of SCF and endogenous TGF- β .

Keywords

prostate; stem cell niche; stem cell factor; transforming growth factor-beta

BACKGROUND

Stem cells in most tissues are located in a specific regulatory environment called the niche, which modulates the survival and proliferation of the cells (1-6). The microenvironmental

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signals that control stem cell fate include secreted factors such as Wnts, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), stem cell factor (SCF), transforming growth factor- β (TGF- β) and leukemia inhibitory factor (LIF), and cell-cell interactions mediated by integral membrane proteins (6,7). Abundant evidence indicates that stem cell homeostasis in the niche is regulated by a balance between inhibitory and mitogenic cytokines. A prostate stem cell niche is located in the proximal region of murine prostatic ducts and is enriched in cells exhibiting the stem cell-like characteristics of quiescence, high proliferative capacity and the ability of single cells to regenerate prostatic ducts (8-11). Stem cells are thought to reside in the basal layer of the acinus (and express cytokeratins 5 and 14), with luminal cells (expressing cytokeratins 8 and 18) arising from differentiating basal cells (12-14). However, recent evidence also indicates the existence of a luminal stem cell that can self-renew and give rise to basal cells (15). We have recently demonstrated that TGF- β is a regulator of prostatic homeostasis. This cytokine differentially controls the proliferation of primitive cells in the proximal region of ducts and more mature cells in the distal region of ducts in an androgen-dependent manner (16). We now show that primitive cells are modulated in their niche by a balance between TGF- β , which inhibits stem cell proliferation and promotes differentiation, and stem cell factor (SCF), which promotes their proliferation and inhibits differentiation.

TGF- β is a well studied multifunctional regulatory polypeptide that controls many aspects of cellular function, including proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis and survival (17). It inhibits the proliferation of normal epithelial cells, which suggests a role in tumor suppression (17). TGF- β also maintains stem cell quiescence in a number of systems, including bone marrow, intestine, mammary gland, liver, epidermis, brain and prostate (1,16,18-25). In the prostate, the stem cell-containing proximal region of prostatic ducts, and cells in the distal region respond differentially to TGF- β . In the androgen-replete animal, active TGF- β maintains stem cells in a quiescent state. After castration, TGF- β levels increase distally, resulting in apoptosis and involution of this region, while TGF- β levels decrease proximally, thus priming the stem cells for response to mitogenic cytokines such as EGF (epidermal growth factor), FGF-2, IGF (insulin-like growth factor) and SCF, which stimulate their division after androgen replacement (16).

The actions of TGF- β depend on several parameters including the presence of other cytokines (17). In many instances the biological effect of TGF- β is modulated by mitogenic cytokines that act to abrogate its inhibitory effect. Convincing evidence indicates that SCF promotes the survival of hematopoietic stem cells and germ cells and counteracts the inhibitory effect of TGF- β on these cells (26-29). The interplay between mitogenic cytokines, such as EGF, FGF-1 and IGF, and inhibitory factors, such as TGF- β , regulate the proliferation of cells in the stem cell-containing bulge region of the hair follicle (30,31), thus regulating the hair cycle. In the gut, irradiation results in an increase in EGF in all regions of the crypt, including its base where the stem cells are located (32,33). This suggests that EGF has a role in increasing proliferation in wounded tissue thus influencing stem cell proliferation. In addition, evidence indicates that a balance between the TGF- β and FGF signaling pathways regulates gut development (21). In the prostate, we have shown that while the proliferation of cells from the stem cell-containing proximal region is inhibited by TGF- β , this inhibition is abrogated by the addition of SCF, FGF-2 or EGF, thus indicating that a balance of positive and negative regulatory signals controls proliferation in the stem cell niche (16).

In this manuscript we show that endogenous TGF- β inhibits cellular proliferation of both proximal and distal cells and that this effect is abrogated by SCF. We suggest that the interaction between the inhibitory TGF- β and the stimulatory SCF controls and modulates the prostate stem cell niche.

MATERIALS AND METHODS

Cell Isolation and Growth Studies

Cells from the proximal and distal regions of prostatic ducts were isolated as described previously (8) and cultured at 10^4 cells/well in collagen (rat tail type I; BD Biosciences, Bedford, MA) gels (34) or at 2×10^3 cells/well in 96-well collagen-coated (Advanced BioMatrix, San Diego, CA) wells. The culture medium was Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, MO) with 10% fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA), and containing transferrin (10 $\mu\text{g/ml}$), hydrocortisone (0.5 $\mu\text{g/ml}$), cholera toxin (10 ng/ml), dihydrotestosterone (10^{-7}M) and phosphoethanolamine (0.1 mM; all reagents from Sigma). Growth factors and antibodies were added to the culture medium 3 hours after cells were seeded and included SCF (100 ng/ml; R&D, Minneapolis, MN), and anti-TGF- β antibodies (25 $\mu\text{g/ml}$ anti-TGF- β 1, TGF- β 2, TGF- β 3, clone 1D11, R&D). Control wells received either no factors or all of the factors. The medium was removed and replaced with fresh medium and factors every two days. Cell growth in each assay was measured and quantified as described previously (8). The numbers of ducts per well and the numbers of branch points emanating from each duct was determined using a phase contrast microscope and scoring individual ducts. Ducts in collagen gels were fixed in 70% ethanol and processed for immunohistochemistry after 10 days of culture. Cells seeded on collagen were fixed after 6 days with ice-cold methanol:acetone (1:1). Experiments were performed in triplicate.

Immunocytochemistry and Immunohistochemistry

Paraffin sections were deparaffinised in xylene, followed by dehydration in ethanol (100% followed by 70%) and rehydration in phosphate buffered saline (PBS). Colonies on collagen were rehydrated with PBS before immunocytochemistry. Basal (CK14) and luminal cytokeratins (CK8) were detected using polyclonal rabbit anti-CK14 (Ab BL18) or mouse anti-CK8 (Ab LE61; gifts of Dr. B. Lane, Dundee, Scotland). Slides were rinsed briefly and were then blocked for one hour at room temperature with 5 % normal goat serum in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Antibodies were added at room temperature for 1 hour. Slides were washed and the secondary antibodies (goat anti-rabbit Texas red or goat anti-mouse FITC – Southern Biotechnology Associates, Birmingham, AL, or goat anti-rat Alexa Fluor 594 – Molecular Probes, Carlsbad, CA) were added at room temperature for 30 minutes. Slides were washed again and mounted in Fluormount G medium (Southern Biotechnology Associates) and examined using a Zeiss Axiophot microscope for evidence of fluorescence.

RESULTS

We have previously shown that proximal and distal cells respond differentially to TGF- β added to the medium, with cells from the proximal region being less inhibited than cells from the distal region (16). In order to determine whether endogenous TGF- β has a role in the proliferation and differentiation of prostate cells, we examined the effects of neutralizing antibodies to this cytokine on proximal and distal cells using two assays, namely the ability of cells to grow on collagen-coated wells and the ability of cells to form ducts when seeded in collagen gels (8,34,35). Seeding cells on collagen-coated films permits a quantitative estimate of cell numbers as well as a quantitative determination of differentiation by determining the numbers of basal and luminal colonies that form. Adding cells to collagen gels allows them to form complete ductal structures, which can be evaluated in terms of numbers and complexity (branch points and size).

Endogenous TGF- β inhibits proliferation and promotes a luminal phenotype in proximal cells on collagen-coated wells

i) Effects on proliferation—Proximal and distal cells were cultured on collagen-coated wells in the absence or presence of SCF and/or anti-TGF- β antibodies to ascertain their effect on cell growth. As noted previously (8), cells isolated from the proximal stem cell containing region have a greater growth capacity than cells obtained from the distal region (Fig. 1). While both proximal and distal cells responded to the addition of SCF and/or the ablation of endogenous TGF- β , proximal cells were twice as sensitive as distal cells to these factors. SCF alone significantly enhanced the growth of both proximal (1.9-fold; $p < 0.01$) and distal cells (1.1-fold, $p < 0.01$) compared to that of untreated cells (Figure 1; Table I). This increase in proximal cell number was significantly higher than the 1.1-fold increase in distal cell number ($p < 0.001$). Ablation of TGF- β activity resulted in a 2.0-fold and a 1.2-fold increase in proximal and distal growth respectively over untreated wells ($p < 0.01$ in both cases). The 2-fold increase in cell number observed in the proximal cells was significantly higher than the 1.2-fold increase observed in the distal cell growth ($p < 0.001$). The addition of SCF in conjunction with the ablation of TGF- β resulted in a 2.4-fold increase ($p < 0.01$) in proximal and a 1.3-fold increase ($p < 0.01$) in distal cell growth over untreated wells (Figure 1; Table I), a significantly different increase in growth of proximal cells compared with that of distal cells ($p < 0.001$). These data illustrate that proliferation of cells in the proximal region of the prostatic duct is modulated by both SCF and endogenous TGF- β . SCF has a mitogenic effect on the cells, while TGF- β inhibits their proliferation, an effect that can be abrogated by inhibiting TGF- β activity. Cells in the distal region respond somewhat similarly to these factors, but are significantly less sensitive to these cytokines than the proximal cells.

ii) Effects on differentiation—We determined the effects of SCF and endogenous TGF- β on differentiation as the addition of TGF- β to a rat prostate basal cell line or to primary murine prostate cells promotes differentiation to a luminal phenotype (13,16) while SCF promotes a primitive phenotype in many types of cells (26,28,36,37). Previous work has indicated that basal cells, include the stem cell population and express cytokeratins 5 and 14, while luminal cells, are more differentiated cells that arise from basal cells and express cytokeratins 8 and 18 (12,14). The addition of SCF to proximal cells resulted in a 1.4-fold increase ($p < 0.01$) in the number of basal colonies (Fig. 2A; Table II) over untreated wells. Ablation of endogenous TGF- β significantly enhanced this effect, with the number of basal colonies increasing 2.0-fold ($p < 0.0001$) over untreated wells. The combination of SCF and TGF- β ablation had an even greater effect on promoting a basal phenotype as the number of basal colonies increased 2.6-fold over that noted for untreated cells ($p < 0.0001$). No differences were noted in the numbers of luminal colonies under any conditions (Figure 2A, Table II). Similar but less pronounced effects were noted in cells isolated from the distal region of ducts. Addition of SCF resulted in a 1.5-fold increase in the number of basal colonies over untreated wells, although the observed difference was not significant (Figure 2B; Table II). Ablation of endogenous TGF- β and the combined effect of SCF and TGF- β also enhanced the number of basal colonies. No differences were noted in the numbers of luminal colonies under any conditions (Fig. 2B).

These results indicate that ablation of endogenous TGF- β and addition of SCF promotes proliferation. It also indicates that TGF- β promotes a luminal phenotype in these cultures. Ablation of endogenous TGF- β alone resulted in an increase in cell number of both proximal and distal cells, an effect further enhanced by the addition of SCF. While both proximal and distal cells respond to these cytokines, proximal cells are more responsive than distal cells. Similarly, ablation of endogenous TGF- β and addition of SCF to proximal cells

promotes a basal phenotype, as indicated by an increase in the number of basal cell colonies compared with luminal cell colonies.

Endogenous TGF- β inhibits duct-forming capacity and promotes a luminal phenotype in prostatic ducts in collagen gels

Although the addition of TGF- β to a rat basal line promotes its differentiation to luminal cells (13) and results in differentiation of primary murine basal prostate cells to a luminal phenotype (16) the effects of endogenous TGF- β on prostatic cell proliferation and differentiation is currently unknown. To study this we seeded prostate cells in collagen gels. The sizes and complexity (branch points) of the resulting ducts is indicative of the proliferative potential of the individual duct-forming cells. The most primitive cells form the largest most complex ducts while cells with less regenerative potential form smaller structures (8).

i) TGF- β inhibits ability of prostatic cells to form ducts—Although SCF alone did not significantly increase the numbers of ducts formed by proximal cells in collagen gels compared with untreated cells (duct number increased 1.2-fold; $p < 0.1$), it did significantly increase both the complexity (i.e., the number of branch points by 2.2-fold; $p < 0.01$) and the size (1.3-fold; $p < 0.01$; Fig. 3A–C Table III) of prostatic ducts formed from proximal and distal cells. However, the inhibition of endogenous TGF- β produced by proximal cells resulted in a striking increase in the numbers of ducts, their complexity and size compared with untreated cells (25.3-fold; $p < 0.001$, 7.3-fold; $p < 0.001$ and 1.5-fold; $p < 0.001$ respectively; Fig. 3A–C; Table III). Ablation of TGF- β activity in distal cells also resulted in increases in duct number (6.2-fold; $p < 0.01$), their complexity (4.3-fold; $p < 0.01$) and size (1.3-fold; $p < 0.1$) compared with untreated distal cells. However, cells isolated from the proximal region were considerably more sensitive to the ablation of endogenous TGF- β than distal cells as the numbers of ducts increased by 25.3-fold compared with a 6.2 fold increase in ducts formed from distal cells ($p < 0.01$). The addition of SCF to cells whose TGF- β activity had also been ablated further increased the duct-forming ability of both proximal and distal cells by a similar magnitude (1.4-fold; $p < 0.01$) compared with the duct-forming ability of those cells lacking only TGF- β activity. These data indicate that both proximal and distal cells have a similar sensitivity to SCF while proximal cells are more sensitive to the inhibitory effects of endogenous TGF- β .

ii) TGF- β promotes luminal cell differentiation in ducts—As the ablation of TGF- β resulted in striking increases in the duct-forming ability of both proximal and distal cells, we determined the effects of its abrogation and of SCF on ductal differentiation as evidenced by the ratio of basal to luminal cells within the prostatic ducts. Ducts from untreated cells contained mixtures of basal and luminal cells (Fig. 4 A, B). The abrogation of TGF- β activity and the addition of SCF had striking effects on the composition of cells in the ducts formed from proximal cells (Fig. 4 C, E, G). These ducts consisted entirely of basal cells with no luminal differentiation being evident. In contrast, although ablating TGF- β increased the numbers of ducts formed from distal cells (see above), no significant effects were noted on their composition (Fig. 4 D, F, H).

These data indicate that endogenous TGF- β and SCF have significant effects on prostate growth and on the cellular composition of ducts. Although endogenous TGF- β inhibits the proliferation of both proximal and distal cells, proximal cells are more sensitive to its inhibitory effects than distal cells. The addition of SCF to cells in which TGF- β activity has been ablated enhances duct-forming ability by an equivalent amount in both proximal and distal cells indicating that cells from both regions respond equivalently to this cytokine. Both SCF and endogenous TGF- β have profound effects on the cellular composition of

ducts arising from proximal cells (TGF- β promotes a luminal phenotype), whereas they have minimal effects on the composition of ducts arising from distal cells.

DISCUSSION

TGF- β is a multifunctional cytokine that normally functions in an autocrine manner to inhibit the proliferation of epithelial, endothelial, smooth muscle and hematopoietic cells (17,38). Loss of autocrine TGF- β activity may provide epithelial cells with a growth advantage, in some cases leading to malignant progression and the development of cancer (17,39). The addition of TGF- β to a rat prostate basal cell line in the absence of mitogens induces differentiation while the presence of mitogens, such as IGF-1, suppresses TGF- β -induced differentiation, promoting a more primitive basal phenotype (40). Similarly Narine et al. (41) demonstrated that either EGF or FGF-2 ablated TGF- β -induced growth inhibition of fibroblast proliferation and FGF-2 inhibited differentiation, as evidenced by loss of alpha smooth muscle actin expression, while Burger et al. (42) demonstrated that FGF-2 maintains erythroid cells in a primitive state while TGF- β promotes their differentiation. SCF and its receptor, c-Kit, have been implicated as regulators of cellular proliferation and differentiation of progenitor cells in a variety of systems, including hematopoiesis, gametogenesis and melanogenesis (26,43,44). Evidence indicates that TGF- β acts with SCF to regulate proliferation in some types of cells, with c-Kit expression being down-regulated by TGF- β and the effect of SCF thus being negated by this factor (45,46). We have previously shown that addition of TGF- β to primary murine proximal and distal cells results in a decrease in the number of basal colonies and an increase in the number of luminal colonies, indicating that TGF- β drives cells from a primitive basal phenotype to a mature luminal phenotype (16). We have also shown that cells in the proximal region of prostatic ducts produce and activate more TGF- β than cells in the distal region (16). Here we show that SCF and endogenous TGF- β have profound effects on the proliferation and cellular composition of prostatic ducts and that endogenous TGF- β inhibits proliferation and promotes a luminal phenotype of cells isolated from the stem cell containing proximal region of prostatic ducts. We find that SCF increases proliferation of prostate cells cultured from proximal and distal regions of prostatic ducts. We also show that the proliferative activity of SCF is enhanced when endogenous TGF- β activity is ablated and that this effect is more pronounced in cells isolated from the stem cell enriched proximal region of ducts than in cells isolated from distal ductal regions. We observed that SCF is produced by prostatic stromal cells surrounding ducts (unpublished observations). In the bone marrow, SCF is similarly produced by stromal cells and binds to c-kit receptors on primitive hematopoietic cells (47). We have been unable to identify clear evidence of c-kit expression on prostate epithelial cells. It is possible that these receptors are present in levels too low to be detected by antibody-based techniques. Cells in culture respond to SCF thus indicating that a c-kit expressing population is present. Gene expression analysis of primitive fetal and adult populations show clear evidence of c-kit in fetal prostate cells whereas adult prostate cells lack c-kit expression (10).

We therefore suggest that proliferative cytokines and TGF- β act in concert to control the proliferation and differentiation of prostate cells. In the proximal stem cell containing region, where cells produce and activate more TGF- β (16) cellular proliferation is inhibited and its effect overrides the effects of growth promoting or differentiation-inhibitory cytokines such as SCF. SCF could also promote the proliferation of basal cells rather than antagonizing their differentiation. However, when TGF- β activity is ablated, cell proliferation is increased and a luminal phenotype is no longer favored (as demonstrated by the presence of basal cells and the absence of luminal cells). In distal cells, while cell proliferation is also affected by TGF- β and SCF in a similar manner (although to a lesser extent), to that observed in proximal cells, basal/luminal cellular composition is not affected

by the cross talk between TGF- β and SCF. The more sensitive response of primitive proximal cells to these factors suggests that differences in the local prostatic environment along the ductal network (proximal to distal) may be critical in controlling prostatic homeostasis.

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Figure 1. The inhibitory effect of TGF- β on cell growth is negated by the addition of SCF and the ablation of endogenous TGF- β by anti- TGF- β antibodies

Cells from the proximal (P) and distal (D) regions of prostatic ducts were seeded on collagen (2×10^3 cells/well) and overlaid with medium containing SCF (100 ng/ml), anti- TGF- β antibodies (25 μ g/ml) or a combination of SCF and anti- TGF- β antibodies. Control wells received medium without additives. Wells were cultured for 6 days, after which cells were enumerated.

Figure 2. Addition of SCF and ablation of endogenous TGF- β promotes a primitive basal phenotype

Cells from the proximal and distal regions of prostatic ducts were seeded on collagen films (2×10^3 cells/well) in the absence and presence of SCF (100 ng/ml), anti- TGF- β antibodies (25 μ g/ml) or a combination of SCF and anti- TGF- β antibodies. Cells were cultured for 6 days, after which colonies were probed with antibodies against basal (CK14) or luminal cytokeratins (CK8).

Figure 3. Endogenous TGF- β inhibits duct-forming capacity in collagen gels

Cells from the proximal (P) and distal (D) regions of prostatic ducts were seeded in collagen gels (10^4 cells/well) in the absence and presence of SCF (100 ng/ml), anti-TGF- β antibodies (25 $\mu\text{g}/\text{ml}$) or a combination of SCF and anti-TGF- β antibodies. Cells were cultured for 10 days before being analyzed for (A) number of ducts, (B) complexity (branch points) and (C) size of ducts.

Figure 4. Addition of SCF and ablation of endogenous TGF- β in proximal cells promotes the formation of ducts with a basal phenotype

Cells from the proximal and distal region of prostatic ducts were seeded in collagen gels (10^4 cells/well) in the absence and presence of SCF (100 ng/ml), anti-TGF- β antibodies (25 μ g/ml) or a combination of SCF and anti-TGF- β antibodies. Cells were cultured for 10 days, after which collagen gels were fixed, sectioned and probed with antibodies against basal (CK14 – red) or luminal cytokines (CK8 – green). Bars = 100 μ m.

Table I

Proximal cells are more sensitive to the inhibitory effects of endogenous TGF- β and the stimulatory effect of SCF than are distal cells.

Location of cells	Treatment	Cell number $\times 10^4$ (mean $\pm$ SD)	Fold increase	P value
Proximal	Untreated	1.38 $\pm$ 0.15		
	+ SCF	2.62 $\pm$ 0.23	1.9	0.01
	+ anti TGF- β Ab	2.76 $\pm$ 0.09	2.0	0.01
	+SCF + anti TGF- β Ab	3.38 $\pm$ 0.08	2.4	0.001
Distal	Untreated	0.37 $\pm$ 0.03		
	+ SCF	0.42 $\pm$ 0.07	1.1	0.01
	+ anti TGF- β Ab	0.46 $\pm$ 0.03	1.2	0.01
	+SCF + anti TGF- β Ab	0.49 $\pm$ 0.03	1.3	0.01

Table II

Ablation of endogenous TGF- β and addition of SCF promotes a primitive basal phenotype in proximal cells.

Location of cells	Colony type	Untreated (mean $\pm$ SD)	+ SCF (mean $\pm$ SD)	+ anti TGF- β Ab (mean $\pm$ SD)	+ SCF + anti TGF- β Ab (mean $\pm$ SD)
Proximal	Basal	9.08 $\pm$ 3.58	12.67 $\pm$ 5.10	18.58 $\pm$ 3.80	23.50 $\pm$ 6.3
	Luminal	5.17 $\pm$ 3.21	4.92 $\pm$ 2.90	5.00 $\pm$ 4.61	4.92 $\pm$ 5.43
Distal	Basal	1.83 $\pm$ 1.81	2.83 $\pm$ 1.64	3.50 $\pm$ 1.93	3.75 $\pm$ 2.73
	Luminal	1.75 $\pm$ 1.42	1.75 $\pm$ 1.54	1.42 $\pm$ 1.16	1.58 $\pm$ 1.98

Table III

Endogenous TGF- β inhibits duct formation in collagen gels.

Location of cells		Untreated (mean $\pm$ SD)	+ SCF (mean $\pm$ SD)	+ anti TGF- β Ab (mean $\pm$ SD)	+ SCF + anti TGF- β Ab (mean $\pm$ SD)
Proximal	Duct number	19.33 $\pm$ 1.13	23.67 $\pm$ 1.87	488.67 $\pm$ 50.5	697.67 $\pm$ 53.5
	Branch points	2.60 $\pm$ 0.51	5.60 $\pm$ 1.08	19.00 $\pm$ 1.31	25.20 $\pm$ 1.20
	Surface area	38.31 $\pm$ 3.2	50.07 $\pm$ 3.05	56.44 $\pm$ 1.76	62.49 $\pm$ 2.23
Distal	Duct number	7.33 $\pm$ 1.44	9.33 $\pm$ 1.44	45.33 $\pm$ 3.18	63.67 $\pm$ 2.99
	Branch points	0.60 $\pm$ 0.25	1.40 $\pm$ 0.51	2.60 $\pm$ 0.51	4.00 $\pm$ 0.63
	Surface area	8.79 $\pm$ 0.50	10.38 $\pm$ 0.42	11.15 $\pm$ 0.52	12.44 $\pm$ 0.33